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(54) Title: METHODS OF DETECTING EARLY RENAL DISEASE IN ANIMALS

(57) Abstract: The present invention provides a method for the detection of early renal disease in animals. The method includes the steps of (a) obtaining a sample from an animal to be tested and (b) determining the amount of albumin in the sample. An amount of albumin in the range of from 10 μg/ml to about 300 μg/ml indicates the presence of early renal disease. The present invention also provides antibodies to canine, feline and equine albumin which can be used to detect the presence of early renal disease.

METHODS OF DETECTING EARLY RENAL DISEASE IN ANIMALS

FIELD OF THE INVENTION

The present invention relates to detection of early renal disease in animals, and more particularly to use of microalbuminuria as a marker of early renal disease.

BACKGROUND OF THE INVENTION

Glomerular disease is a broad term used to describe a number of renal diseases that can lead to renal failure and death. Damage to the glomerulus increases capillary permeability to proteins such as albumin, resulting in the presence of proteins in urine (referred to as proteinuria).

In humans, proteinuria can result from a number of diseases, including diabetes, hypertension and IgA nephropathy. The conventional test for proteinuria in humans is to use a standard protein dipstick assay as described, for example, in Bakris, *Curr. Opin. in Neph. and Hypertension*, 5:219-223 (1996). Dipsticks that are chemically impregnated with sulfosalicylic acid to measure proteins in a sample are commercially available, for example from Boehringer-Mannheim, Germany (Chemstrips™) and Ames Co., USA (Albustix™). One drawback to these dipstick assays is that they require a significant amount of protein in the urine to be detected. Amounts of protein in humans of less than 300 milligrams per day are not detectable by the dipstick assay, yet proteinuria may still be present. Another drawback to these protein-based assays is that they are incapable of discriminating between different types of protein (e.g., albumin, globulin, etc.) that may be present in urine. Proteinuria may result from the leakage of serum proteins into glomerular filtrate due to glomerulernephritis; however, proteinuria may also be present due to problems unrelated to renal disease such as bladder infections or a high-protein diet.

Lower amounts of albumin in the urine, referred to as "microalbuminuria," indicate a level of albumin that is greater than in normal patients, but lower than in patients with overt proteinuria, i.e., clinically proteinuric. In humans, microalbuminuria refers to amounts of albumin between 30 milligrams per day and 300 milligrams per day according to Watts, *Clin. Chem.*, 32(8):1544-1548 (1986). Methods to detect human microalbuminuria are known and include those methods that use an anti-human albumin antibody to detect amounts of human albumin that are not detectable by known dipstick methods. Such methods of detecting human

5   microalbuminuria are described, for example, in U.S. Patent No. 5,246,835, issued
       on September 21, 1993, to Suzuki et al.

              Although microalbuminuria can be detected in humans, the utility of
       detecting microalbuminuria in humans may be very limited, at least according to
       some reports. For example, using the microalbuminuria tests to predict renal disease
10   has only been recommended for humans with diabetes according to Bakris, supra.
       Because disorders other than diabetes, such as hypertension, heart disease and IgA
       nephropathy do not lead to consistent microalbuminuria in humans, according to
       Bakris, supra, detecting microalbuminuria has poor predictive value for later renal
       disease associated with these non-diabetic disorders states. Accordingly, using
15   microalbuminuria tests to screen for potential or early renal disease in non-diabetic
       human patients is generally not recommended by Bakris, supra.

              Renal disease is also a significant health problem in companion animals,
       particularly dogs and cats. In dogs, the primary cause of renal disease is damage to
       the glomerulus in the kidney. Although glomerular damage in dogs can occur in any
20   number of ways, it is most commonly caused when circulating immune complexes
       (i.e., antibody/antigen complexes) are deposited in the glomerular capillaries as a
       result of systemic illness as described in Batamuzi, et al., *Vet Record*, 143;16-20
       (1988). Several diseases have been implicated in the pathogenesis of immune
       complex formation, including for example, dirofilariasis and other parasitic
25   infections, diabetes, hypothyroidism and others.

              Early renal disease in veterinary medicine has been characterized by
       glomerular changes detectable by histopathology, including the use of light
       microscopy or occasionally immunofluorescence as reported in Vaden, *Proc. 17th*
       ACVIM, 420 (1999). However, as reported in that paper, these techniques can lead to
30   misdiagnosis of the cause of the renal disease. Determining the cause of the renal
       disease is useful in formulating an appropriate treatment regimen. For example, if
       the cause of the renal disease is immune-mediated, then immunosuppressive therapy
       may be appropriate. However, currently available assays to detect human
       microalbuminuria are not sufficiently sensitive to detect canine microalbumunuria.
35   Thus, a need exists for assays to detect canine early renal disease in
       companion animals. The present invention satisfies this need and provides related
       advantages as well.

SUMMARY OF THE INVENTION

The present invention relates to a method and kit for the detection of early renal disease in animals. Preferred animals to test for early renal disease are companion animals with dogs, cats and horses being the most preferred. Method and kit embodiments disclosed herein are based on the discovery that the presence of albumin in a sample from an animal, in the range of 10 µg/ml to 300 µg/ml can be used as an indicator of early renal disease. The most preferred sample to test is urine although any sample that is useful for measuring leakage of albumin from the glomerulus can be used. Any assay capable of detecting albumin may be used in the instant method or kit although preferred methods and kits employ immunologically-based assays, preferably single-step assays. The most preferred assay is an immunologically-based assay utilizing an anti-albumin antibody.

The present invention also provides isolated antibodies which can be used in detecting albumin levels in animal samples. Any antibody which binds albumin from the test animal can be used; preferred antibodies bind canine albumin and/or feline albumin and/or equine albumin. Preferred antibodies are TNB1, TNB3, TNB4, TNB5, TNB6, H352, H386, H387, H388, H389, H390, H391, H393, H394, H395, H396, H397, H398, H399, H400, H401, and H402. Also included are cultured cells which produce antibodies suitable for practicing the present invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention generally relates to a novel method of detecting early renal disease in animals and to novel antibodies that selectively bind to albumin from one or more specie of animal. More particularly, the present invention relates to the discovery that the presence of microalbuminuria can be used to predict early renal disease in animals, particularly immune-mediated renal diseases. Therefore, the methods can also be useful for prescribing a treatment for an animal. Suitable treatment can be designed to delay or prevent the onset of late-stage renal disease. Examples of such treatment include, for example, pharmacological or dietary modification. The present invention is also useful in monitoring the effectiveness of a prescribed treatment.

A method of the present invention can be generally accomplished by:

(a) obtaining a sample from an animal; and

(b) determining the amount of albumin in the sample.

5   An amount of albumin in a range of from about 10μg/ml to about 300 μg/ml in the
        sample is indicative of early renal disease.

                It is to be noted that the term "a" entity or "an" entity refers to one or more of
        that entity. For example, a protein refers to one or more proteins or at least one
        protein. As such, the terms "a" "an" "one or more" and "at least one" can be used
10   interchangeably herein. The terms "comprising," "including," and "having" can also
        be used interchangeably. In addition, the terms "amount" and "level" are also
        interchangeable and may be used to describe a concentration or a specific quantity.
        Furthermore, the term "selected from the group consisting of" refers to one or more
        members of the group in the list that follows, including mixtures (i.e. combinations)
15   of two or more members.

                As used herein, the term "renal disease" is defined as a dysfunction of the
        glomerular filtration process. Glomerular dysfunction may be transient or it may be
        chronic, depending on the underlying cause of the disease. One consequence of
        glomerular dysfunction is that proteins which are normally retained in the blood, leak
20   through the glomerulus, into the glomerular filtrate and eventually into the urine.
        One example of a protein which may be present in urine due to glomerular
        dysfunction is albumin and its presence in urine at low levels has been termed
        microalbuminuria. The term "microalbuminuria," as used herein, refers to an amount
        of albumin that is present in a sample in a range from about 10 μg/ml to about 300
25   μg/ml when the sample is normalized to a specific gravity of 1.010 grams/milliliter
        (g/ml). This is greater than the amount found in healthy animals which is typically
        low, i.e., less than 10 μg/ml. Microalbuminuria may arise as a consequence of
        damage to the kidney resulting from, for example, immune-complex-mediated
        glomerulernephritis. As used herein, the term "late-stage renal disease" is used to
30   define a state in which an animal has lost 70% or more of its renal function, with
        corresponding, elevated levels in the animal's serum metabolites, in particular blood-
        urea nitrogen (BUN) and serum creatinine levels. As used herein, the term "early
        renal disease" is defined as the presence of microalbuminuria in an animal in the
        absence of detectable changes in renal function (i.e. increased BUN, serum creatinine
35   or decreased ability to concentrate urine). As such, an albumin level in a sample
        ranging from about 10 μg/ml to about 300 μg/ml when the sample is normalized to a
        specific gravity of 1.010 g/ml is indicative of early renal disease.

5   As used herein, the term "animal" is meant to encompass any non-human organism capable of developing early renal disease. Suitable animals to test for microalbuminuria include, but are not limited to companion animals (i.e. pets), food animals, work animals, or zoo animals. Preferred animals include, but are not limited to, cats, dogs, horses, ferrets and other Mustelids, cattle, sheep, swine, and rodents.

10   More preferred animals include cats, dogs, horses and other companion animals, with cats, dogs and horses being even more preferred. As used herein, the term "companion animal" refers to any animal which a human regards as a pet. As used herein, a cat refers to any member of the cat family (i.e., Felidae), including domestic cats, wild cats and zoo cats. Examples of cats include, but are not limited to,

15   domestic cats, lions, tigers, leopards, panthers, cougars, bobcats, lynx, jaguars, cheetahs, and servals. A preferred cat is a domestic cat. As used herein, a dog refers to any member of the family Canidae, including, but not limited to, domestic dogs, wild dogs, foxes, wolves, jackals, and coyotes and other members of the family Canidae. A preferred dog is a domestic dog. As used herein, a horse refers to any

20   member of the family Equidae. An equid is a hoofed mammal and includes, but is not limited to, domestic horses and wild horses, such as, horses, asses, donkeys, and zebras. Preferred horses include domestic horses, including race horses.

       In one embodiment of the present invention, a sample is obtained, or collected, from an animal to be tested for microalbuminuria. The animal may or may

25   not be suspected of having early stage renal disease. A sample is any specimen obtained from the animal that can be used to measure albumin leakage from the glomerulus. A preferred sample is a bodily fluid that can be used to measure albumin leakage from the glomerulus. Those skilled in the art can readily identify appropriate samples.

30   Urine is particularly suitable as the sample. Urine samples can be collected from animals by methods known in the art, including, for example, collecting while the animal is voiding, or collecting by catheterization, or by cystocentesis. Urine may be refrigerated or frozen before assay, but is preferably assayed soon after collection.

35   Although not necessary for the present invention, the sample may be pre-treated as desired. For example, the sample can be normalized to a desired specific gravity. Normalizing the sample by appropriate dilution methods known in the art

5   permits quantification of microalbuminuria independent of the concentration (e.g.
       specific gravity) of the sample. Although any desired specific gravity can be readily
       selected by those skilled in the art, a particularly suitable specific gravity is 1.010. If
       another specific gravity value is desired for normalizing a sample, those skilled in the
       art can readily determine the appropriate albumin amounts that would fall within the
10   definition of microalbuminuria for the desired specific gravity.

              After obtaining the sample, the level of albumin in that sample is determined.
       As used herein, the terms "determine," "determine the level of albumin," "determine
       the amount of albumin," "determine the albumin level," and the like are meant to
       encompass any technique which can be used to detect or measure the presence of
15   albumin in a sample. Albumin is an example of an analyte. The term "analyte, as
       used herein, is used to describe any molecule or compound present in a sample.
       Such techniques may give qualitative or quantitative results. Albumin levels can be
       determined by detecting the entire albumin protein or by detecting fragments,
       degradation products or reaction products of albumin. In a preferred method, the
20   level of albumin is determined using a suitable albumin-binding compound.

              As used herein, the terms "albumin-binding molecule", "albumin-binding
       compound", "anti-albumin compound", and the like are used interchangeably and
       refer to any molecule which binds to albumin and forms a stable complex. A
       preferred albumin-binding compound is one which selectively binds albumin from an
25   animal. The term "selectively binds albumin" means to preferentially bind to
       albumin as opposed to binding other proteins unrelated to albumin. A particularly
       useful albumin-binding compound is a anti-albumin antibody. As used herein, the
       terms "anti-albumin antibody," "antibody to albumin," "antibody to animal
       albumin," "antibody having specificity for albumin from animals," " animal albumin
30   antibody," and the like refer to an antibody that preferentially binds albumin from
       one or more animals. A particularly suitable anti-albumin antibody preferentially
       binds to canine, feline and/or equine albumin as opposed to binding to different,
       unrelated canine, feline or equine proteins. Another particularly suitable anti-
       albumin antibody preferentially binds to canine albumin as opposed to binding to a
35   different, unrelated canine protein. Another particularly suitable antibody to
       companion animal albumin preferentially binds to feline albumin as opposed to
       binding to a different, unrelated feline protein. Another particularly suitable

5 antibody to companion animal albumin preferentially binds to equine albumin as
opposed to binding to a different, unrelated equine protein.

The present invention also includes isolated (i.e., removed from their natural
milieu) antibodies that selectively bind to albumin of one ore more animal species.
Isolated antibodies of the present invention can include antibodies in serum, or

10 antibodies that have been purified to varying degrees. Antibodies of the present
invention can be polyclonal or monoclonal, or can be functional equivalents such as
antibody fragments and genetically-engineered antibodies, including single chain
antibodies or chimeric antibodies that can bind to one or more epitopes on albumin.
A suitable method to produce antibodies effective for use in the present invention

15 includes (a) administering to an animal an effective amount of a protein, peptide or
mimetope thereof to produce the antibodies and (b) recovering the antibodies.
Antibodies raised against defined proteins or mimetopes can be advantageous
because such antibodies are not substantially contaminated with antibodies against
other substances that might otherwise cause interference in a diagnostic assay.

20 Methods to produce such antibodies are known in the art and are described in detail
in Harlow et al., *Antibodies, a Laboratory Manual* (Cold Spring Harbor Labs Press,
1988), and include immunizing animals to produce preparations of polyclonal
antibodies that are recovered from, for example, ascites fluid and purified by
methods known in the art to yield preparations that are reactive to animal albumin.

25 Many species have proteins sharing closely related sequences and therefore it may be
difficult using standard immunization protocols to produce antibodies which
recognize a protein from only one specie. Therefore, modification of standard
methods used to produce antibodies, such as, for example, subtractive hybridization
techniques, are also contemplated. Such modifications can be those known to those

30 skilled in the art or additionally modified techniques as disclosed within this
application. In another method, antibodies for use in the present invention are
produced recombinantly using techniques disclosed in Sambrook et al., *Molecular
Cloning: A Laboratory Manual*, (Cold Spring Harbor Labs Press, 1989).

As noted previously, other suitable methods include producing monoclonal

35 antibodies. Briefly, monoclonal antibodies are produced from the fusion of spleen
cells from an immunized animal and myeloma cells to produce a hybridoma.
Hybridomas can be screened for production of the proper antibody, then cultured and

5   the antibodies harvested. As used herein, the term "cultured cell" refers to

hybridomas or any cell which produces an antibody. Methods to produce and screen

such hybridomas are described in Harlow, et al., *supra*. Methods to prepare an

antigen so that antibodies produced will be reactive with animal albumin are known

in the art and are described, for example, in Harlow, et al., *supra*. Preparation of the

10   antigen material for injection into the animal includes any technique known in the

art, and include, for example, using the full-length protein, using peptides selected

from immunogenic regions of the protein, modifying the antigen by methods such as,

for example, dinitrophenol coupling, arsynyl coupling, denaturation of the antigen,

coupling antigen to protein carriers such as, for example, keyhole limpet

15   hemacyanin, peptides containing Class II- T-cell receptor binding sites, to beads, and

any other method known in the art. See Harlow, et al., *supra*.

The anti-albumin antibodies of the present invention can include

multifunctional antibodies, for example a bifunctional antibody having at least one

functional portion that specifically binds to animal albumin. Such multifunctional

20   antibodies can include, for example, a chimeric molecule comprising a portion of the

molecule that binds to animal albumin and a second portion that enables the chimeric

molecule to be bound to a substrate or to be detected in such a manner that the

binding to the albumin is unimpaired. Examples of suitable second portions include

but are not limited to a fragment of an immunoglobulin molecule, a fluorescent

25   protein or an enzyme.

In addition to anti-albumin antibodies, albumin-binding molecules can also

include proteins and peptides that bind to albumin. Such proteins and peptides may

be from natural, recombinant or synthetic sources and may or may not be purified.

Examples of non-antibody, albumin-binding, proteins include, but are not limited to,

30   the 42-kilodalton (kDa) Protein A from *Staphlococcus aureus*, Protein G from *S.

aureus* and *Eschericia coli*, the rat 60-kDa albumin binding protein (gp60) and the

human renal tubule cubilin protein. The use of functional homologues of such

proteins, from these or other species, for the detection of albumin is also

contemplated. Hybrids or fusions of albumin-binding proteins which retain their

35   albumin-binding ability may also be used. In such hybrids, the albumin-binding

portion of the protein would be joined to a second portion which allows the hybrid to

be bound to a substrate or to be detected. Examples of suitable second portions

5   include, but are not limited to, a fragment of an immunoglobulin molecule, an

epitope tag, a fluorescent protein or an enzyme.

        An albumin-binding molecule used in the present invention can be contained

in a formulation. For example, an antibody can be combined with a buffer in which

the antibody is solubilized, and/or with a carrier. Suitable buffers and carriers are

10   known to those skilled in the art. Examples of suitable buffers include any buffer in

which an albumin-binding molecule can function to selectively bind to albumin, such

as, but not limited to, phosphate buffered saline, water, saline, phosphate buffer,

HEPES buffer (N-2-hydroxyethylpiperazine-N'-2-ethansulfonic acid buffered saline)

TES buffer (Tris -EDTA buffered saline), Tris buffer and TAE buffer (Tris-acetate-

15   EDTA). Examples of carriers include, but are not limited to, polymeric matrices,

toxoids, and serum albumins, such as bovine serum albumin. Carriers can be

combined with an albumin-binding molecule or conjugated (i.e. attached) to an

albumin-binding molecule in such a manner as to not substantially interfere with the

ability of the albumin-binding molecule to selectively bind to albumin. In addition,

20   suitable formulations of the present invention can include not only the albumin-

binding molecule to specie-specific albumin, but also one or more additional

antigens or antibodies useful for detecting albumin.

        As used herein, the term "contacting" refers to the introduction of a sample

putatively containing albumin to an albumin-binding compound, for example, by

25   combining or mixing the sample with the albumin-binding compound. When

albumin is present in the sample, an albumin-compound complex is then formed;

such complex formation refers to the ability of an anti-albumin compound to

selectively bind to the albumin in order to form a stable complex that can be

detected. Detection can be qualitative, quantitative, or semi-quantitative. Binding

30   albumin in the sample to the albumin-binding compound is accomplished under

conditions suitable to form a complex. Such conditions (e.g., appropriate

concentrations, buffers, temperatures, reaction times) as well as methods to optimize

such conditions are known to those skilled in the art. Binding can be measured using

a variety of methods standard in the art including, but not limited to, enzyme

35   immunoassays (e.g., ELISA), immunoprecipitations, immunoblot assays and other

immunoassays as described, for example, in Sambrook et al., *supra*, and Harlow, et

al., *supra*. These references also provide examples of complex formation conditions.

In one embodiment, an albumin/albumin-binding compound complex, also referred to herein as an albumin-compound complex, can be formed in solution. In another embodiment, an albumin/albumin-binding compound complex can be formed in which the albumin or the albumin-binding compound is immobilized on (e.g., coated onto) a substrate. Immobilization techniques are known to those skilled in the art. Suitable substrate materials include, but are not limited to, plastic, glass, gel, celluloid, fabric, paper, and particulate materials. Examples of substrate materials include, but are not limited to, latex, polystyrene, nylon, nitrocellulose, agarose, cotton, PVDF (poly-vinylidene-fluoride), and magnetic resin. Suitable shapes for substrate material include, but are not limited to, a well (e.g., microtiter dish well), a microtiter plate, a dipstick, a strip, a bead, a lateral flow apparatus, a membrane, a filter, a tube, a dish, a celluloid-type matrix, a magnetic particle, and other particulates. Particularly preferred substrates include, for example, an ELISA plate, a dipstick, an immunodot strip, a radioimmunoassay plate, an agarose bead, a plastic bead, a latex bead, a sponge, a cotton thread, a plastic chip, an immunoblot membrane, an immunoblot paper and a flow-through membrane. In one embodiment, a substrate, such as a particulate, can include a detectable marker. For descriptions of examples of substrate materials, see, for example, Kemeny, D.M. (1991) *A Practical Guide to ELISA*, Pergamon Press, Elmsford, NY pp 33-44, and Price, C. and Newman, D. eds. *Principles and Practice of Immunoassay*, 2nd edition (1997) Stockton Press, NY, NY, both of which are incorporated herein by reference in their entirety.

In a preferred embodiment, an anti-albumin compound is immobilized on a substrate, such as a microtiter dish well, a dipstick, an immunodot strip, or a lateral flow apparatus. A sample collected from an animal is applied to the substrate and incubated under conditions suitable (i.e., sufficient) to allow for anti-albumin compound-albumin complex formation bound to the substrate (i.e., albumin in the sample binds to the anti-albumin compound immobilized on the substrate).

In accordance with the present invention, once formed, an albumin-binding molecule /albumin complex is detected. As used herein, the term "detecting complex formation" refers to identifying the presence of albumin-binding compound complexed to albumin. If complexes are formed, the amount of complexes formed can, but need not be, quantified. Complex formation, or selective binding, between a

putative albumin-composition with an albumin-binding compound can be measured
(i.e., detected, determined) using a variety of methods standard in the art (see, for
example, Sambrook et al. *supra.*), examples of which are disclosed herein. A
complex can be detected in a variety of ways including, but not limited to use of one
or more of the following assays: an enzyme-linked immunoassay, a competitive
enzyme-linked immunoassay, a radioimmunoassay, a fluorescence immunoassay, a
chemiluminescent assay, a lateral flow assay, a flow-through assay, an agglutination
assay, a particulate-based assay (e.g., using particulates such as, but not limited to,
magnetic particles or plastic polymers, such as latex or polystyrene beads), an
immunoprecipitation assay, a BioCore™ assay (e.g., using colloidal gold), an
immunodot assay (e.g., CMG's Immunodot System, Fribourg, Switzerland), and an
immunoblot assay (e.g., a western blot), an phosphorescence assay, a flow-through
assay, a particulate-based assay, a chromatography assay, a PAGe-based assay, a
surface plasmon resonance assay, a spectrophotometric assay and an electronic
sensory assay. Such assays are well known to those skilled in the art.

Assays can be used to give qualitative or quantitative results depending on
how they are used. The assay results can be based on detecting the entire albumin
molecule or fragments, degradation products or reaction products of albumin. Some
assays, such as agglutination, particulate separation, and immunoprecipitation, can be
observed visually (e.g., either by eye or by a machines, such as a densitometer or
spectrophotometer) without the need for a detectable marker.

In other assays, conjugation (i.e., attachment) of a detectable marker to the
anti-albumin compound or to a reagent that selectively binds to the anti-albumin
compound aids in detecting complex formation. A detectable marker can be
conjugated to the anti-albumin compound or reagent at a site that does not interfere
with ability of the anti-albumin compound to bind albumin. Methods of conjugation
are known to those of skill in the art. Examples of detectable markers include, but
are not limited to, a radioactive label, a fluorescent label, a chemiluminescent label, a
chromophoric label, an enzyme label, a phosphorescent label, an electronic label; a
metal sol label, a colored bead, a physical label, or a ligand. A ligand refers to a
molecule that binds selectively to another molecule. Preferred detectable markers
include, but are not limited to, fluorescein, a radioisotope, a phosphatase (e.g.,
alkaline phosphatase), biotin, avidin, a peroxidase (e.g., horseradish peroxidase),

5   beta-galactosidase, and biotin-related compounds or avidin-related compounds (e.g.,
        streptavidin or ImmunoPure® NeutrAvidin).

                In one embodiment, an animal albumin-compound complex can be detected
        by contacting a sample with a specific compound-antibody conjugated to a detectable
        marker. A detectable marker can be conjugated to an anti-albumin antibody or other

10   compound which binds the albumin-binding-compound in such a manner as not to
        block the ability of the anti-compound antibody or other compound to bind to the
        canine albumin-binding compound being detected. Preferred detectable markers
        include, but are not limited to, fluorescein, a radioisotope, a phosphatase (e.g.,
        alkaline phosphatase), biotin, avidin, a peroxidase (e.g., horseradish peroxidase),

15   beta-galactosidase, and biotin-related compounds or avidin-related compounds (e.g.,
        streptavidin or ImmunoPure® NeutrAvidin).

                In another embodiment, a complex is detected by contacting the complex
        with an indicator molecule. Suitable indicator molecules include molecules that can
        bind to the albumin/ albumin-binding molecule complex or to the albumin. As such,

20   an indicator molecule can comprise, for example, an albumin-binding reagent, such
        as an antibody. Preferred indicator molecules that are antibodies include, for
        example, antibodies reactive with the antibodies from species of animal in which the
        anti- albumin antibodies are produced. An indicator molecule itself can be attached
        to a detectable marker of the present invention. For example, an antibody can be

25   conjugated to biotin, horseradish peroxidase, alkaline phosphatase or fluorescein.

                The present invention can further comprise one or more layers and/or types of
        secondary molecules or other binding molecules capable of detecting the presence of
        an indicator molecule. For example, an untagged (i.e., not conjugated to a detectable
        marker) secondary antibody that selectively binds to an indicator molecule can be

30   bound to a tagged (i.e., conjugated to a detectable marker) tertiary antibody that
        selectively binds to the secondary antibody. Suitable secondary antibodies, tertiary
        antibodies and other secondary or tertiary molecules can be readily selected by those
        skilled in the art. Preferred tertiary molecules can also be selected by those skilled in
        the art based upon the characteristics of the secondary molecule. The same strategy

35   can be applied for subsequent layers.

                Preferably, the indicator molecule is conjugated to a detectable marker. A
        developing agent is added, if required, and the substrate is submitted to a detection

5   device for analysis. In some protocols, washing steps are added after one or both

       complex formation steps in order to remove excess reagents. If such steps are used,

       they involve conditions known to those skilled in the art such that excess reagents are

       removed but the complex is retained.

             One embodiment to detect microalbuminuria involves the use of a lateral

10   flow assay, examples of which are described in U.S. Patent No. 5,424,193, issued

       June 13, 1995, by Pronovost et al.; U.S. Patent No. 5,415,994, issued May 16, 1995,

       by Imrich et al; WO 94/29696, published December 22, 1994, by Miller et al.; and

       WO 94/01775, published January 20, 1994, by Pawlak et al. A lateral flow assay is

       an example of a single-step assay. In a single-step assay, once the sample has been

15   obtained and made ready for testing, only a single action is necessary on the part of

       the user to detect the present of an analyte. For example, the sample, in whole or

       part, can be applied to a device which then measures analyte in the sample. In one

       embodiment, a sample is placed in a lateral flow apparatus that includes the

       following components: (a) a support structure defining a flow path; (b) a labeling

20   reagent comprising a bead conjugated to a specific antibody, the labeling reagent

       being impregnated within the support structure in a labeling zone; and (c) a capture

       reagent. Preferred antibodies include those disclosed herein. The capture reagent is

       located downstream of the labeling reagent within a capture zone fluidly connected

       to the labeling zone in such a manner that the labeling reagent can flow from the

25   labeling zone into the capture zone. The support structure comprises a material that

       does not impede the flow of the beads from the labeling zone to the capture zone.

       Suitable materials for use as a support structure include ionic (i.e., anionic or

       cationic) material. Examples of such a material include, but are not limited to,

       nitrocellulose, PVDF, or carboxymethylcellulose. The support structure defines a

30   flow path that is lateral and is divided into zones, namely a labeling zone and a

       capture zone. The apparatus can further include a sample receiving zone located

       along the flow path, preferably upstream of the labeling reagent. The flow path in the

       support structure is created by contacting a portion of the support structure

       downstream of the capture zone, preferably at the end of the flow path, to an

35   absorbent capable of absorbing excess liquid from the labeling and capture zones.

             In another embodiment, a lateral flow apparatus used to detect albumin

       includes: (a) a support structure defining a flow path; (b) a labeling reagent

5   comprising a anti- albumin antibody as described above, the labeling reagent

impregnated within the support structure in a labeling zone; and (c) a capture

reagent, the capture reagent being located downstream of the labeling reagent within

a capture zone fluidly connected to the labeling zone in such a manner that the

labeling reagent can flow from the labeling zone into the capture zone. The

10   apparatus preferably also includes a sample receiving zone located along the flow

path, preferably upstream of the labeling reagent. The apparatus preferably also

includes an absorbent located at the end of the flow path. One preferred embodiment

includes a capture reagent comprising anti-canine albumin antibody.

Once the albumin level has been measured, an assessment of whether early

15   renal disease is present can then be made. Assessing the presence of early renal

disease means comparing the level of albumin in the test sample to the level found in

healthy animals. The presence of microalbuminuria in the sample, in the absence of

changes in renal function, is indicative of early renal disease. As used herein, the

term "indicative of early renal disease" is means sufficient glomerular dysfunction is

20   present to allow albumin to pass into the urine in the range of from about 10 µg/ml to

about 300 µg/ml. The amount of albumin present in the sample may vary depending

on the amount of damage present but in early renal disease, the albumin level is

higher than that found in healthy animals but lower than that detectable by current

methods used to measure proteinuria. In the present invention, a determination of

25   early renal disease is made when the level of albumin in the sample is determined to

be in the range of from about 10 µg/ml to about 300 µg/ml. The upper range of

albumin levels can also be about 25 µg/ml, about 50 µg/ml, about 75 µg/ml, about

100 µg/ml, about 125 µg/ml, about 150 µg/ml, about 175 µg/ml, about 200 µg/ml,

about 225 µg/ml, about 250 µg/ml, about 275 µg/ml, or about 300 µg/ml. The level

30   of albumin in the sample may vary depending on the severity of the damage to the

kidney. Preferred embodiments of the present inventions can detect albumin when

about 10%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%,

about 80%, or about 90% of kidney function is lost. A more preferred embodiment

can detect microalbuminuria in time for medical intervention which may then delay

35   or prevent the onset of late-stage renal disease. Such intervention may, for example,

include, but is not limited to the use of pharmacological compounds or dietary

modifications to delay or prevent the progression of renal disease.

One embodiment of the present invention is a "dipstick" device which can detect microalbuminuria in animals. Dipsticks may be constructed in a variety of ways that partly depend on the way in which they will be used. They may be held directly in a sample (e.g., a urine stream), dipped directly in sample contained in a collection vessel, or have sample applied to a strip contained in a plastic cassette or platform. Another example of a dipstick is a "flow-through" device, an example of which is a heterogenous immunometric assay system based on a capture antibody immobilized onto a membrane attached to an absorbent reservoir. A "bead" refers to a particulate substrate composed of a matrix such as latex or polystyrene, which can be covalently or non-covalently cross-linked to a detection molecule. A preferred embodiment of the "dipstick" assay is an immunometric system, described in U.S. Patent No. 5,656,502, issued on August 12, 1997, to MacKay and Fredrickson, and U.S. Patent No. 6,001,658, issued December 14, 1999 to Fredrickson, both incorporated herein by reference. Particularly preferred is an ImmunoDip™ device available from Diagnostic Chemicals Ltd., PEI, CA.

Non-immunological methods may also be used. In order to detect microalbuminuria, methods such as preconcentration of the urine in order to concentrate albumin may be used to increase sensitivity of the test to protein. Such non-immunological methods include, for example, urine electrophoresis, where detection of microalbuminuria can be determined by methods known in the art, and include, for example, protein staining. In another embodiment, a protein based albumin test may be used to determine microalbuminuria on a preconcentrated sample of urine from an animal.

The methods of the present invention can be used to detect nephropathy in a canid, felid, equid, or other animal, particularly when the nephropathy is glomerulonephropathy, and especially glomerulonephritis. More specifically, the microalbuminuria measurement is correlated to the presence of early renal disease in a target animal. As used herein, the term "nephropathy" and/or "renal disease" refers to any disease of the kidneys, and may include, for example, nephritis of the glomerular, tubular, or interstitial renal tissues.

Such early stage nephropathy can result from many different causes, including, for example, allergy, cancer, parasitic, viral, or bacterial infection of any tissue in the animal, exposure to renal toxins, immune-mediated diseases, such as

5   systemic lupus erythematosus and vasculitis, malignancy, Vitamin D3 rodenticides,

pyelonephritis, leptospirosis, urinary tract obstruction, chronic inflammatory disease,

pyoderma, pancreatitis, prostatitis, immune-mediated diseases, dental disease, high

blood pressure, or diabetes. As used herein, an "infectious agent" is one that infects

animals and include, but are not limited to, viruses, bacteria, fungi, endoparasites and

10   ectoparasites. Examples of viral infectious agents include, but are not limited to,

adenoviruses, caliciviruses, coronaviruses, distemper viruses, hepatitis viruses,

herpesviruses, immunodeficiency viruses, infectious peritonitis viruses, leukemia

viruses, oncogenic viruses, papilloma viruses, parainfluenza viruses, parvoviruses,

rabies viruses, and reoviruses, as well as other cancer-causing or cancer-related

15   viruses. Examples of bacterial infectious agents include, but are not limited to,

Actinomyces, Bacillus, Bacteroides, Bartonella, Bordetella, Borrelia, Brucella,

Campylobacter, Capnocytophaga, Clostridium, Corynebacterium, Coxiella,

Dermatophilus, Ehrlichia, Enterococcus, Escherichia, Francisella, Fusobacterium,

Haemobartonella, Helicobacter, Klebsiella, L-form bacteria, *Leptospira, Listeria,*

20   *Mycobacteria, Mycoplasma, Neorickettsia, Nocardia, Pasteurella, Peptococcus,*

Peptostreptococcus, Proteus, Pseudomonas, Rickettsia, Rochalimaea, Salmonella,

Shigella, Staphylococcus, Streptococcus, and *Yersinia.* Examples of fungal

infectious agents include, but are not limited to, *Absidia, Acremonium, Alternaria,*

Aspergillus, Basidiobolus, Bipolaris, Blastomyces, Candida, Chlamydia,

25   *Coccidioides, Conidiobolus, Cryptococcus, Curvalaria, Epidermophyton, Exophiala,*

Geotrichum, Histoplasma, Madurella, Malassezia, Microsporum, Moniliella,

Mortierella, Mucor, Paecilomyces, Penicillium, Phialemonium, Phialophora,

Prototheca, Pseudallescheria, Pseudomicrodochium, Pythium, Rhinosporidium,

Rhizopus, Scolecobasidium, Sporothrix, Stemphylium, Trichophyton, Trichosporon,

30   and *Xylohypha.* Examples of protozoan parasite infectious agents include, but are

not limited to, *Babesia, Balantidium, Besnoitia, Cryptosporidium, Eimeria,*

Encephalitozoon, Entamoeba, Giardia, Hammondia, Hepatozoon, Isospora,

Leishmania, Microsporidia, *Neospora, Nosema, Pentatrichomonas, Plasmodium,*

Pneumocystis, Sarcocystis, Schistosoma, Theileria, Toxoplasma, and *Trypanosoma.*

35   Examples of helminth parasite infectious agents include, but are not limited to,

Acanthocheilonema, Aelurostrongylus, Ancylostoma, Angiostrongylus, Ascaris,

Brugia, Bunostomum, Capillaria, Chabertia, Cooperia, Crenosoma, Dictyocaulus,

5   *Dioctophyme, Dipetalonema, Diphyllobothrium, Diplydium, Dirofilaria,*

Dracunculus, Enterobius, Filaroides, Haemonchus, Lagochilascaris, Loa,

Mansonella, Muellerius, Nanophyetus, Necator, Nematodirus, Oesophagostomum,

Onchocerca, Opisthorchis, Ostertagia, Parafilaria, Paragonimus, Parascaris,

Physaloptera, Protostrongylus, Setaria, Spirocerca, Spirometra, Stephanofilaria,

10   *Strongyloides, Strongylus, Thelazia, Toxascaris, Toxocara, Trichinella,*

Trichostrongylus, Trichuris. Uncinaria, and *Wuchereria.* Examples of ectoparasite

infectious agents include, but are not limited to, fleas, ticks, including hard ticks and

soft ticks, flies such as midges, mosquitoes, sand flies, black flies, horse flies, horn

flies, deer flies, tsetse flies, stable flies, myiasis-causing flies and biting gnats, ants,

15   spiders, lice, mites, and true bugs, such as bed bugs and kissing bugs.

The present invention may also be used to measure multiple analytes. Other

analytes may be any analyte which can be detected in sample suitable for use in

detecting early renal disease. Additional analytes can be used to detect, for example,

infectious disease or inborn errors of metabolism.

20   The present invention also relates to antibodies that bind to albumin from an

animal being tested. A preferred antibody is one which detects albumin levels when

the amount in the sample is about 50μg/ml, more preferably 25 μg/ml, more

preferably 10 μg/ml. Another preferred antibody is one which detects albumin levels

when the amount in the sample is about 50μg/ml, more preferably about25 μg/ml,

25   more preferably about 10 μg/ml and the detection method is a dipstick device

described in U.S. Patent No. 6, 001, 658. A preferred antibody is one which

competes with any of the monoclonal antibodies TNB1, TNB3, TNB4, TNB5,

TNB6, H352, H386, H387, H388, H389, H390, H391, H393, H394, H395, H396,

H397, H398, H399, H400, H401, or H402 for selective binding to animal albumin,

30   preferably canine albumin. Another preferred embodiment is an antibody which

binds to the same or related epitope, as defined by sequence homology, bound by the

antibodies TNB3, TNB6 and H402. A preferred antibody is selected from the group

consisting of TNB1, TNB3, TNB4, TNB5, TNB6, H352, H386, H387, H388, H389,

H390, H391, H393, H394, H395, H396, H397, H398, H399, H400, H401, and H402

35   . More preferred is an antibody selected from the group consisting of TNB3, TNB6

and H402. As used herein, the terms "compete" and "inhibit selective binding" refer

5   to the ability of an antibody to prevent another antibody from binding to the same protein as described in the included examples.

The present invention also includes kits suitable for detecting animal albumin using the methods disclosed herein. Suitable means of detection include the techniques disclosed herein, utilizing compounds that bind the desired animal

10   albumin, such as, for example, an anti- albumin antibody. As such, a kit can also comprise a detectable marker, such as an antibody that selectively binds to the albumin binding compound or other indicator molecules. The kit can also contain associated components, such as, but not limited to, buffers, labels, containers, inserts, tubings, vials, syringes and the like.

15   The present invention is based on a surprising discovery that microalbuminuria in canids can be used as a marker to predict the development of renal disease in nondiabetic dogs as well as diabetic dogs because microalbuminuria does not clearly have predictive value in nondiabetic human patients. Similar uses are contemplated in other animals. However, despite this surprising discovery, until

20   the present invention, effective methods to detect microalbuminuria in dogs did not exist. Conventional human microalbuminuria detection methods do not detect dog microalbuminuria as described in the examples below.

The following examples are provided for the purposes of illustration and are not intended to limit the scope of the present invention.

25   Example 1

Measurement of Microalbuminuria in Normal, CRF and ARF Dogs

Urine samples were collected from 134 canine patients at the Colorado State University Teaching Hospital. These samples included urine from normal dogs, dogs suffering chronic renal failure, dogs suffering acute renal failure, and proteinuric dogs

30   without renal failure. Samples were frozen at -20°C for at least 24 hours and then thawed prior to use. Albumin levels were quantified by a microradial immunodiffusion assay as described in McDonald, Weber & Thiele, "Construction and Use of a Template Block for Radial Immunodiffusion" *Anal Biochem* 186:165-168 (1990) using a commercial anti-albumin antibody (polyclonal rabbit anti-dog

35   albumin, available from Nordic Immunology distributed by Accurate Chemical and Scientific Corp., Westbury, N.Y.). For this assay the antibody at 1.5% (vol/vol) was added to melted 0.75% (wt/vol) EEO agarose in PBS. Gels, at a thickness of 0.75 mm

5   were poured between two glass plates. The gels were allowed to solidify and after

one of the glass plates was removed, allowed to dry slightly. Acrylic blocks,

described in McDonald et al., *supra.*, were placed on the agarose and 5 µl sample or

standard was placed in each well of the acrylic block. Samples were run either

undiluted or if the resulting ring was too large to measure the sample was diluted and

10   re-tested. The standard curve using dog albumin (dog albumin fraction V, available

from Sigma, St. Louis, MO) was linear within the range of 10 - 100 µg/ml. The

acrylic blocks were left on the agarose and the unit was placed in a moist chamber and

incubated overnight at room temperature. The agarose gels were then soaked in

distilled water for several hours to remove the excess protein from the gel, the gel was

15   dried and then stained with Coomassie Brilliant Blue so that the precipitin rings could

be readily visualized. The diameter of each ring was measured and the ring diameter

from each sample was compared to the standard curve and the albumin concentration

of each sample was calculated.

The advantage of using this system for measuring albumin in the urine is that

20   this system is more sensitive that the traditional assay with wells cut into the gels.

This increased sensitivity is related to the to the concentrated delivery of the antigen

into a small area as opposed to the larger surface area created by the edges of a well

cut into the agarose For this initial study, samples that had less than or equal to 50

µg/ml were deemed normal, samples that had levels between 51 and 300 µg/ml were

25   deemed microalbuminuric, and those that had levels over 300 µg/ml were deemed

macroalbuminuric. The results of this study are shown in Table 1.

Table 1. Urinary albumin levels in 134 canine urine samples

Albumin Level	Number of Animals	Percentage
Normal (0-50 µg/ml)	59	44%
Microalbuminuria (51-300µg/ml)	21	16%
Macroalbuminuria (>300µg/ml)	54	40%

30   Example 2

ELISA Quantification of Microalbuminuria

Rabbit anti-canine serum albumin IgG (anti-CSA IgG) is diluted to 375 ng/ml

in coating buffer (50 mM Na_2CO_2 /$NaCHO_3$, pH 9.6). The diluted anti-CSA IgG

solution is added to a plate of MaxiSorp™ C8 Break-apart Microwells (Nunc Cat. #

5   473768) at 100 μl/well, covered and incubated overnight (16 to 24 hours) at 4°C.
The plate is washed four times with phosphate buffered saline with 0.05% Tween 20
(PBS-T) in an automatic plate washer and blotted dry. Blocking buffer (StabilCoat™
available from Surmodics Cat. #SC01-1000) is added at 200 μl/well, covered and
incubated at room temperature for at least 1 hour.

10   While blocking, the canine serum albumin (CSA) dilution series is prepared.
First, the CSA is diluted to 120 ng/ml in assay diluent (0.1% casein hydrolysate in
PBS-T). This solution is serially diluted (1 part to 1 part) to make 60 ng/ml, 30
ng/ml, 7.5 ng/ml, 3.75 ng/ml, and 1.875 ng/ml. The last 5 standards are used for the
standard curve (30 ng/ml and less) along with a "zero" standard (assay diluent with

15   no CSA). Each urine sample to be tested is diluted 1/500, 1/1000, 1/2000, 1/4000,
1/8000, 1/16000 and 1/32000 in assay diluent.

The plate is then washed four times in an automatic plate washer and blotted
dry. The CSA standard and diluted urine sample are added at 100 μl/well of each to
the test wells. Assay diluent is added to duplicate wells for background control. The

20   plate is covered and incubated for 2 hours at room temperature. As previously, the
plate is washed four times with PBS-T and blot dry.

Dilute biotin labeled goat anti-CSA IgG (Bethyl Laboratories, Cat. #E40-
113)] to 125 ng/ml in assay diluent. Add 100 μl/well of diluted biotin labeled goat
anti-CSA IgG to all test wells. Cover plate and incubate for 30 minutes at room

25   temperature. As previously, wash the plate four times with PBS-T and blot dry.

Dilute horseradish peroxidase labeled streptavidin (KPL Cat.# 14-30-00) to
500 ng/ml (1/1000 dilution) in assay diluent and add to all test wells at 100 μl/well.
Cover and incubate at room temperature for 30 minutes. As previously, wash the
plate four times with PBS-T and blot dry.

30   Mix TMB microwell peroxidase 2 component system (KPL Cat.#50-76-03)
solutions together at equal volumes and add 100 μl/well of the TMB mixture to all
wells. Cover and incubate for 30 minutes at room temperature. The reaction is
stopped by adding 100 μl/well of stop solution (1M H_3PO_4) directly to the TMB in
each well. Read the wells at 450 nm in a spectrophotometer. Average the values of

35   all duplicate wells, if any, and subtract background value from all the test values.
Generate a standard curve from the standard values and generate a regression line
(r^2>0.95). Using the regression formula, compute the CSA (ng/ml) value for each

5 sample and multiply this value by the dilution factor. Only those values that fall in

the linear portion of the standard curve should be used.

Example 3

Use of the ImmunoDip™ stick for the Detection of Microalbuminuria in Canine

Urine

10   Three ImmunoDip™ sticks (product number 700-01) for the detection of

microalbuminuria in humans, were obtained from Diagnostic Chemicals Limited,

Charlottetown, Prince Edward Island, Canada. Two canine urine samples (numbered

1086 and 1098) were selected from a group of samples obtained from dogs at the

Colorado State University Veterinary Teaching Hospital, Fort Collins, Colorado.

15 Samples 1086 and 1098 were selected based on their albumin-levels as determined

by an in-house ELISA to detect microalbuminuria in dogs. Sample 1086 was a

negative sample, and sample 1098 had an albumin concentration of 221 µg/ml. For a

positive control, approximately 50 µl of human blood was added to 5 ml deionized

water

20   Measurement of albumin in the urine was performed following the

manufacturer's directions. Briefly, 3 ml of urine or the blood-spiked water was

added to a test tube. The ImmunoDip stick was removed from the pouch and placed

in the test tube containing the urine making sure the fluid level was above the vent

hole in the device. The device was left in the sample for a minimum of 3 minutes

25 after which it was removed and read by comparing the relative intensities of the two

bands according to the interpretation-of-results insert that accompanies the test kit.

The results of the in-house ELISA and the ImmunoDip tests are shown in Table 2.

Table 2. ImmunoDip™ stick for microalbuminuria results

Sample	In-House ELISA	ImmunoDip
1086	0 µg/ml	Negative
1098	221 µg/ml	Negative
Blood-spiked Water	Not-tested	Positive

30

   The limit of detection in the ImmunoDip test for human urine albumin is 12

µg/ml. Sample 1098 contained canine urine at a level significantly above this lower

limit yet was negative for albumin by the ImmunDip™ test. These data suggest that

5   this device does not recognize canine albumin, at least not in order to detect
microalbuminuria.

Example 4

Use of Micral® Test Strips for the Detection of Microalbuminuria in Canine Urine

Fourteen Micral® urine test strips (product number 417146) for the detection

10   of microalbuminuria in humans, were obtained from Roche BMC, Indianapolis,
Indiana. Thirteen canine urine samples were selected from a group of samples
obtained from employee's dogs. Samples for use were selected based on their
albumin-levels as determined by an in-house ELISA to detect microalbuminuria in
dogs. Samples 2A, 4A & 16 A were negative samples while the remaining samples

15   had albumin concentrations ranging in value from 31.3 to >650 µg/ml. As a positive
control, 50 µl of human blood was added to 5 ml deionized water.

Measurement of albumin in the urine was performed following the
manufacturer's directions. Briefly, each dog's urine was collected in a sample
collection cup. In addition, blood-spiked water was placed in a test tube. The·

20   Micral® stick was removed from the vial and placed in the collection cup (or test tube
containing the blood-spiked water) making sure the fluid level was above the devices
two black lines in each case. The device was left in sample for 5 seconds, removed
and allowed to sit horizontally for 1 minute. The result was determined by
comparing the color of the test pad to the color scale on the vial in accordance with

25   the result insert that accompanied the test. The results of the in-house ELISA and the
Micral® test are shown in Table 3.

The detection limit in the Micral® test for human albumin is about 20 µg/ml.
Several samples contained canine albumin levels significantly above this lower limit
yet were negative for albumin by the Micral® test. These data suggest that this

30   device does not recognize canine urine albumin, at least not in order to detect
microalbuminuria.

Table 3. Micral® urine test strip results

Sample	In-house ELISA	Micral®
1A	79.4 µg/ml	Negative
2A	3.9 µg/ml	Negative
4A	5.9 µg/ml	Negative
5A	35.9 µg/ml	Negative

9A	48.6 µg/ml	Negative
15A	69.4 µg/ml	Negative
16A	8.3 µg/ml	Negative
29A	119.1 µg/ml	Negative
86A	31.3 µg/ml	Negative
87A	65.2 µg/ml	Negative
14	>650 µg/ml	Negative
19	Positive	Negative
45	650 µg/ml	Negative
Blood-spiked water	Not tested	Positive

Example 5

Use of the ImmunoDip™ stick for the Detection of Microalbuminuria in Canine
Urine

Fourteen ImmunoDip™ sticks (product number 700-01) for the detection of
microalbuminuria in humans, were obtained from Diagnostic Chemicals Limited,
Charlottetown, PE, Canada. Thirteen canine urine samples were selected from a
group of samples obtained from dogs that were apparently normal. Samples for use
were selected based on their albumin levels as determined by an in-house ELISA to
detect microalbuminuria in dogs. Samples 2A, 4A &16 A were negative samples
while the remaining samples had albumin concentrations ranging in value from 31.3
to >650 µg/ml. As a positive control, 50 µl of human blood was added to 5 ml
deionized water.

Measurement of albumin in the urine was performed following the
manufacturer's directions. Briefly, 3 ml of urine or the blood-spiked water was
added to a test tube. The ImmunoDip stick was removed from the pouch and placed
in the test tube containing the urine making sure the fluid level was above the
device's vent hole in each case. The device was left in the sample for a minimum of
3 minutes after which, it was removed and read by comparing the relative intensities
of the two bands according to the interpretation-of-results insert that accompanies the
test kit. The results of the in-house ELISA and the ImmunoDip tests are shown in
Table 4.

Table 4. ImmunoDip™ Stick for Microalbuminuria results

Sample	In-house ELISA	ImmunoDip™
1A	79.4 µg/ml	Negative

2A	3.9 µg/ml	Negative
4A	5.9 µg/ml	Negative
5A	35.9 µg/ml	Negative
9A	48.6 µg/ml	Negative
15A	69.4 µg/ml	Negative
16A	8.3 µg/ml	Negative
29A	119.1 µg/ml	Negative
86A	31.3 µg/ml	Negative
87A	65.2 µg/ml	Negative
14	>650 µg/ml	Negative
19	Positive	Negative
45	650 µg/ml	Negative
Blood-spiked water	Not tested	Positive

The detection limit in the ImmunoDip™ test for human albumin is about 20 µg/ml. Several samples contained canine albumin levels significantly above this lower limit yet were negative for albumin by the ImmunoDip™ test. These data suggest that this device does not recognize canine urine albumin, at least not in order to detect microalbuminuria.

Example 6

Use of Micral® Test Strips for the Detection of Microalbuminuria in Canine Urine

Five Micral® urine test strips (product number 417146) for the detection of microalbuminuria in humans were obtained from Roche BMC, Indianapolis, Indiana. Thirteen canine urine samples were selected from a group of samples obtained from dogs that were apparently normal. Samples for use were selected based on their albumin-levels as determined by an in-house ELISA to detect microalbuminuria in dogs. Samples 7 and 12 were negative samples while samples 14 and 25 had albumin levels of 621 µg/ml and >650 µg/ml, respectively. As a positive control, 50 µl of human blood was added to 5 ml deionized water.

Measurement of albumin in the urine was performed following the manufacturer's directions. Briefly, each dog's urine was collected in a sample collection cup. For the positive control, blood-spiked water was placed in a test tube. The Micral® stick was removed from the vial and placed in the collection cup (or test tube containing the blood-spiked water) making sure the fluid level was above the devices two black lines in each case. The device was left in sample for 5 seconds, removed and allowed to sit horizontally for 1 minute. The result was determined by comparing the color of the test pad to the color scale on the vial in accordance with

5   the result insert that accompanied the test. The results of the in-house ELISA and the

Micral® test are shown in Table 5.

Table 5. Micral® urine test strip results

Sample	In–House ELISA	Micral®
7	2.1 µg/ml	Negative
12	0.8 µg/ml	Negative
14	621 µg/ml	Negative
25	>650 µg/ml	Negative
Blood-spiked water	Not tested	Positive

10

The limit of detection in the Micral® test for human urine albumin is about

20 µg/ml. Samples 14 and 25 contained canine albumin levels significantly above

these lower levels yet were negative for albumin by the Micral® test. These data

suggest that this device does recognize canine urine albumin, at least not in order to

15   detect microalbuminuria.

Example 7

Prevalence of Microalbuminuria in dogs

For this study, two separate populations were examined. One sample

population was derived from clinically normal dogs (n=86). The second sample

20   population was derived from Colorado State University Teaching Hospital patients

(n=150) presented for routine health screening, elective procedures, as well as

evaluation of health problems. Microalbuminuria was quantitated using an antigen

capture ELISA. The results of this measurement were normalized to a specific

gravity of 1.010 to account for varying urine concentrations. Albumin in the urine of

25   the hospital patients was also tested using Petstix 8™ urine protein test strips (Idexx

Cat.# 98-06959-00).

Of the 86 clinically normal dogs, 68 (79%) had normalized albumin

concentrations <1.0 mg/dL, 16 (19%) had normalized albumin concentrations >1.0

mg/dL and <30.0 mg/dL , and 2(2%) had normalized albumin concentrations >30.0

30   mg/dL. Of the 159 hospital patients, 112 (70%) were urine test strip negative and 51

of the 112 (46%) test-strip negative samples had normalized albumin concentrations

>1.0 mg/dL. Conversely, 19 of 80 (24%) of samples with <1.0 mg/Dl albumin were

positive on urine test strip (see Table 6).

5

Table 6

Normalized Urine Albumin Concentrations (# of samples)	Urine Protein Test Strip Result (n=159)			
	Neg. (112)	Trace (20)	1+ (15)	2-4+ (12)
<1.0 mg/ dL (80)	61 (54%)	12 (60%)	5 (33%)	2 (17%)
>1.0 and <30.0 mg/ dL (58)	49 (44%)	6 (30%)	2 (13%)	1 (8%)
>30.0 mg/dL (21)	2 (2%)	2 (10%)	8 (53%)	9 (75%)

In the two populations examined, prevalence of microalbuminuria (>1.0

mg/dL and <30.0 mg/ dL) ranged from 19% to 36%. From these results, it appears

10   microalbuminuria is prevalent in a significant number of dogs. Furthermore, use of

commercially available urine protein test strips for the detection of albuminuria yields

a substantial number of false positive results.

Example 8

Purification of canine serum albumin

15   This Example discloses a method for producing canine serum albumin.

Canine serum was adjusted to 50% (w/v) ammonium sulfate, the solution rocked for 3

hours at 4°C, and the insoluble material precipitated by centrifugation at 10,000 x g

for 30 minutes. The supernatant was removed and dialyzed into 25 mM Tris, pH 8.0.

The soluble material was loaded onto a pre-equilibrated, Hi-Trap Q-Sepharose

20   column (Pharmacia, Peapack, NJ) and the proteins eluted using a linear gradient of 0

to 1.0 M NaCl over 25 column volumes (CV). Collected fractions were analyzed by

SDS-PAGe and fractions containing canine albumin were pooled and stored until

needed. Using this method, 414 mg of albumin was purified from 20 ml of canine

serum. Protein sequencing confirmed the purified protein was canine albumin.

25   Example 9

Production of anti-canine albumin antibodies

This example discloses the method used to produce monoclonal antibodies

(Mabs) TNB1, TNB2, TNB3, TNB4, TNB5, TNB6 which recognize canine serum

albumin (CSA).

30   Balb/C mice were immunized by subcutaneous injection with Complete

Freunds Adjuvant mixed with either 25 μg, 50 μg or 100 μg of canine serum albumin

(available from Sigma, St. Louis, MO). After four weeks, blood samples were

obtained and anti-CSA antibody titers determined by ELISA. Based on this data, the

three mice immunized with 100 μg of CSA were chosen for further use in producing

5   hybridomas. Two of these mice were given intravenous (IV) injections containing

100 μg of CSA and the third mouse received 100 μg intraperitoneally. Three days

later, the mice were euthanized, the spleens removed and depleted of T-cells and the

spleen cells fused with SP2/0 mouse myeloma cells following standard protocols.

Individual hybridoma colonies were tested for the production of MAbs which

10   recognize CSA and positive colonies were expanded and dilution cloned until stable

MAb secreting lines were established.

Example 10

Production of anti-canine albumin antibodies using subtractive hybridization

        This Example discloses procedures utilizing subtractive hybridization

15   techniques to produce monoclonal antibodies (Mabs) which recognize canine serum

albumin (CSA).

        Anti-canine CSA hybridoma cell lines were produced using the following,

published method of subtractive hybridization. Balb/C mice were injected

intraperitoneally with 1.0 mg of BSA Fraction V (available from Boehringer

20   Manheim, Indianapolis, IN), followed by IP injections of cyclophosphamide

(CY)(100 mg/kg) at 10 minutes, 24, and 48 hours post-BSA injection. This BSA/CY

treatment was repeated two weeks later. After another two weeks, the mouse was

given a subcutaneous (SC) injection containing 100 μg of CSA (produced as

described in Example 8) mixed with Complete Freunds Adjuvant. After an additional

25   two weeks had passed, blood samples were obtained and serum antibody titers against

CSA and BSA were determined by ELISA. A second injection of CSA (100 μg) was

then given intraperitoneally to boost the animals anti-CSA antibody titers. Two

weeks later, the mouse was given an intravenous (IV) injection of CSA (50 μg) and

after three days, the mouse was sacrificed, its splenocytes harvested and fused with

30   mouse SP2/0 myeloma cells using polyethylene glycol (PEG) following standard

procedures. Individual hybridoma colonies were tested for the production of MAbs

which recognize CSA and positive colonies were expanded and dilution cloned until

stable MAb secreting lines were established. This procedure resulted in the

production of hybridoma lines H398 and H399.

35   In addition to the hybridoma cells lines produced by the above procedure, the

following modified subtractive hybridization procedure was used to produce

additional anti-CSA hybridoma cell lines. 30 μg of CSA (produced as described in

5   Example 8) were injected into the footpad of a Balb/C mouse. Three months later, the

mouse was given an intraperitoneal (IP) injection containing 30 μg of CSA. Four

months after the IP injection, the mouse was given a second IP injection containing

1.0 mg of BSA, followed by IP injections of cyclophosphamide (CY)(100 mg/kg) at

10 minutes, 24, and 48 hours post-BSA injection. After two weeks, this BSA/CY

10   treatment was repeated and after two more weeks had elapsed, the mouse was given a

subcutaneous (SC) injection of CSA (100 μg) mixed with complete Freunds adjuvant.

After another two weeks, blood samples were obtained and serum antibody titers

against CSA and BSA were determined by ELISA. The mouse was then given an

intravenous (IV) injection of CSA (50 μg) and three days later, the mouse was

15   euthanized, its splenocytes harvested and fused with mouse SP2/0 myeloma cells

using polyethylene glycol (PEG) following standard procedures. Individual

hybridoma colonies were tested for the production of MAbs which recognize CSA

and positive colonies were expanded and dilution cloned until stable MAb secreting

lines were established. This protocol resulted in the production of hybridoma cell

20   lines H384, H385, H386, H387, H388, H389, H390, H391, H392, H393, H394, H395,

H396, H400, H401 and H402.

Example 11

Detection of canine serum albumin by ELISA

This example discloses the use of a solid-phase ELISA to test the ability of the

25   anti-canine serum albumin (CSA) antibodies to detect CSA.

The wells of a microtiter plate were coated with CSA (50 μg/well) (produced

as described in Example 8) in carbonate buffer (50 mM carbonate/bicarbonate, pH

9.6) and the plate stored overnight at 4°C. The following day, excess liquid was

removed, the plate blotted dry, and 150 μl of Blocking buffer (0.1% casein in PBS

30   containing 0.05% Tween-20) were added to each well. The plate was incubated at

room temperature (RT) for 30 minutes, after which, the Blocking buffer was removed

and 50 μl of hybridoma supernatant (either undiluted or diluted in blocking buffer)

were added to each well. Following a one hour incubation at RT, the wells were

washed twice using Wash buffer (PBS containing 0.05% Tween-20), 50 μl of HRP-

35   conjugated, goat, anti-mouse IgG and IgM (available from KPL Labs, Gaithersburg,

MD) were added to each well and the plate incubated at RT for 30 minutes. The wells

were washed twice with Wash buffer, and 50 μl of TMB Substrate System (available

5   from KPL Labs) were added to each well. The plate was incubated at RT for 10

minutes after which, the reaction was stopped by the addition of 50 μl of 2N sulfuric

acid to each well. The plate was read at 450 nM using an ELISA plate reader and the

results are shown below in Table 7.

10   Table 7.

Antibody	Undiluted	1:10	1:100
TNB1	1288	852	326
TNB3	1242	1263	922
TNB4	1449	1431	1546
TNB5	1528	1585	1478
TNB6	1782	1436	1103
H386	1274	1273	1187
H387	1394	1369	1326
H388	1485	1529	1408
H389	1685	1646	1265
H390	1558	892	250
H391	1490	1325	916
H393	1744	1603	1640
H394	435	955	577
H395	1265	1049	1001
H396	1564	1773	1390
H397	49	59	48
H398	1822	1641	1501
H399	775	144	64
H400	1572	1610	1239
H401	1839	1683	1511
H402	1799	1752	1447

Example 12

Detection of albumin from several species by ELISA

15   This example demonstrates the ability of three anti-canine albumin

monoclonal Abs to recognize bovine (BSA), canine (CSA), equine (HSA) or human

(HuSA) serum albumin by ELISA using the protocol outlined in Example 11 with the

exception the wells were coated with 3X serial dilutions (from 5 μg/ml to 0.002g/ml)

of the indicated albumin. In addition, 10 μg of the indicated antibody was used in

20   each well. The results are shown in Table 8.

5   Table 8.

	TNB3			
	Coat Protein			
Albumin Concentration (µg/ml)	BSA	CSA	HSA	HuSA
5	.62	3.53	1.67	.12
1.667	.52	3.50	1.37	.15
.556	.43	3.51	.87	.17
.185	.57	3.43	.34	.16
.062	.20	3.14	.19	.15
.021	.17	2.08	.16	.16
.007	.17	.35	.13	.13
.002	.11	.20	.09	.10

	TNB6			
	Coat Protein			
Albumin Concentration (µg/ml)	BSA	CSA	HSA	HuSA
5	.18	3.68	.90	1.69
1.667	.42	3.59	.69	.78
.556	.30	3.59	.52	.47
.185	.24	3.43	.32	.23
.062	.22	3.17	.26	.26
.021	.21	2.36	.22	.22
.007	.20	1.18	.21	.22
.002	.22	.55	.21	.23

	H402			
	Coat Protein			
Albumin Concentration (µg/ml)	BSA	CSA	HSA	HuSA
5	.41	3.41	.87	.97
1.667	.40	3.35	.71	.59
.556	.38	3.31	.57	.41
.185	.35	3.23	.42	.37
.062	.32	2.98	.36	.34
.021	.35	2.10	.32	.31
.007	.35	1.20	.18	.31
.002	.35	.65	.32	.33

5   This data demonstrates mAb's TNB3, TNB6 and H402 have a much greater

affinity for CSA as compared with BSA, HSA or HuSA.

Example 13

Competition ELISA using the anti-albumin mAb's H402, TNB3 and TNB6

          This example compares the ability of the H402, TNB3 and TNB6 monoclonal

10   antibodies to compete for binding to canine serum albumin (CSA). Competition

between antibodies was measured by coating an entire ELISA plate with CSA, adding

a labeled primary antibody to all the wells of the plate and then measuring the ability

of several unlabeled antibodies to compete with the primary antibody for binding to

the CSA. (All primary antibodies were labeled using biotin available from Pierce

15   Chemical, Rockford, IL according to the manufacturers instructions). In this manner,

each plate was used to test the ability of a single primary antibody to compete with

two other anti-albumin antibodies for the ability to bind CSA. In addition, antibody

raised against the extracellular domain of human high affinity IgE receptor alpha

chain (anti-FceRIα) was used on each plate as a negative control. The details of the

20   assay are as follows:

          Three ELISA plates were coated overnight at 4°C with CSA at 1μg/ml. The

following day, the wells were washed using Wash buffer (PBS + 0.05% Tween-20)

and blocked with Blocking Solution (STABILCOAT® IMMUNOASSAY

STABILIZER; available from SurModics, Inc., Eden Prairie, Minnesota) according to

25   the manufacturer's directions. The wells were then washed using Wash buffer, and

100 μl of a single, labeled, primary antibody, either H402 at 20 ng/ml, TNB3 at 8

ng/ml or TNB6 at 12 ng/ml (concentrations were adjusted using Dilution buffer (0.1%

casein in PBS + 0.05% Tween-20)) were added to all of the wells of an individual

plate so that each plate held a different primary antibody. To one row of wells on

30   each plate was then added 100 μl of unlabeled secondary antibody, either H402,

TNB3, TNB6 or anti-HuFCeR1 at 20 μg/ml. Two-fold serial dilutions were then

performed, diluting each secondary antibody across the plate so that the final

concentrations of secondary antibody were from 10 ug/ml to 9 ng/ml. The plates

were incubated at room temperature (RT) for 2 hours, washed with Wash buffer and

35   100 μl of horse-radish-peroxidase conjugated Streptavidin (diluted 1:1000 in Dilution

buffer) were added. Following a 1 hour incubation at RT, the wells were washed with

Wash buffer and 100 μl of developing solution (TMB Substrate; available from KPL

5   Labs, Gaithersburg, MD) were added to each well. After a 30 minute RT incubation, the plates were read at 450 nm using an ELISA plate reader. The results of this assay are shown below in Table 9.

Table 9.

H402 as Primary Antibody			
	Competing (secondary) Antibody		
Antibody Concentration (ng/ml)	H402	TNB3	TNB6	Anti-HuFCER1
10000	.069	2.292	.087	2.584
5000	0.164	2.328	0.195	2.576
2500	0.271	2.341	0.300	2.551
1250	0.517	2.275	0.559	2.569
625	1.212	2.255	1.093	2.592
312.5	2.104	2.262	1.683	2.540
156.25	2.548	2.293	2.239	2.557
78.125	2.670	2.381	2.402	2.512
39.06	2.752	2.461	2.514	2.518
19.53	2.765	2.427	2.655	2.660
9.77	2.798	2.657	2.611	2.639
0	2.710	2.641	2.577	2.642

10

TNB3 as Primary Antibody			
	Competing (secondary) Antibody		
Antibody Concentration (ng/ml)	H402	TNB3	TNB6	Anti-HuFCER1
10000	2.295	0.090	2.290	2.479
5000	2.493	0.245	2.409	2.645
2500	2.445	0.395	2.247	2.508
1250	2.480	0.796	2.185	2.397
625	2.485	1.534	2.239	2.428
312.5	2.378	2.084	2.208	2.483
156.25	2.529	2.535	2.324	2.484
78.125	2.463	2.643	2.351	2.497
39.06	2.566	2.674	2.390	2.509
19.53	2.607	2.740	2.520	2.602
9.77	2.763	2.716	2.611	2.669
0	2.867	2.798	2.756	2.764

TNB6 as Primary Antibody			
	Competing (secondary) Antibody		
Antibody Concentration	H402	TNB3	TNB6	Anti-HuFCER1

(ng/ml)				
10000	0.122	2.307	0.134	2.490
5000	0.303	2.410	0.310	2.604
2500	0.459	2.177	0.473	2.569
1250	0.769	2.276	0.733	2.550
625	1.446	2.283	1.383	2.501
312.5	2.126	2.319	2.053	2.402
156.25	2.502	2.430	2.358	2.564
78.125	2.647	2.455	2.480	2.516
39.06	2.743	2.496	2.557	2.530
19.53	2.745	2.579	2.605	2.582
9.77	2.787	2.697	2.654	2.559
0	2.772	2.685	2.319	2.377

5

The data demonstrate that the monoclonal antibodies H402 and TNB6 compete for binding of canine serum albumin consistent with these antibodies sharing the same, or closely related, epitopes. The data further demonstrate that binding of canine serum albumin by TNB3 is unaffected by H402 or TNB6.

10 Example 14

Binding of canine and feline albumin by H352, H398 and TNB3
This example compares the ability of three anti-albumin antibodies (H352, H398 & TNB3) to bind canine (CSA) or feline (FSA) albumin.

The binding assay was performed as follows. To enable detection, horse-
15 radish peroxidase (HRP) (Pierce Chemical, Rockford, IL) was conjugated to either CSA or FSA following manufacturer's protocol. The wells of a microtiter plate were coated with a range (from 10μg/ml to 9.77 ng/ml) of antibody (either H352, H398 or TNB3) in carbonate buffer (50 mM carbonate/bicarbonate, pH 9.6) and the plates stored overnight at 4°C. The following day, excess liquid was removed and the wells
20 were blocked using blocking solution (STABILCOAT® IMMUNOASSAY STABILIZER; available from SurModics, Inc., Eden Prairie, Minnesota) following manufacturer's instructions. Following removal of the blocking solution, the wells were rinsed using Wash buffer (PBS containing 0.05% Tweeen-20) and HRP-FSA (diluted 1:400 in carbonate buffer) or HRP-CSA (diluted 1:800 in carbonate buffer)
25 were added to the wells and the plate incubated at room temperature (RT) for 30 minutes. The HRP-albumin conjugate was removed, the wells washed twice using Wash Buffer and 50μl of TMB Substrate System (available from KPL Labs, Gaithersburg, MD) were added to each well. The plate was incubated at RT for 10 minutes after which the reaction was stopped by the addition of 50 μl of 2N sulfuric

5   acid to each well. The plate was read at 450 nM using an ELISA plate reader. The

results are shown below in Table 10.

Table 10.

MAb Concentration (ng/ml)	mAb					
	H352		H398		TNB3	
	Coat Protein					
	FSA	CSA	FSA	CSA	FSA	CSA
10000	4.200	4.184	2.984	3.887	0.055	4.191
5000	4.200	4.200	1.944	2.806	0.047	4.184
2500	4.189	4.160	1.532	2.333	0.049	4.177
1250	4.127	4.200	1.187	1.941	0.099	4.186
625	2.740	4.084	0.493	0.769	0.043	4.178
312.5	1.266	2.814	0.168	0.282	0.045	3.410
156.25	0.713	1.598	0.095	0.135	0.043	2.400
78.13	0.324	0.859	0.078	0.090	0.042	1.109
39.06	0.178	0.413	0.053	0.063	0.043	0.543
19.53	0.107	0.236	0.047	0.055	0.049	0.309
9.77	0.077	0.132	0.050	0.051	0.059	0.191
0	0.044	0.048	0.048	0.061	0.049	0.048

10   The data monoclonal antibody H352 binds to both FSA and CSA with roughly equal

affinity. Monoclonal antibody H398 also recognizes both FSA and CSA although it

has greater affinity for CSA. Finally , the data demonstrates that monoclonal

antibody TNB3 binds specifically binds to CSA and does not bind FSA.

Example 15

15   Albumin in canines suffering heartworm-induced renal disease

This example discloses dislcoses the albumin levels present in the canine

Dirofilaria immitis-induced nephropathy. In this model, animals are infected with *D.

immitis* which results in renal damage due to antigen-antibody-complex induced

damage of the glomerulus as described in Grauer, G.F., et. al., *American Journal or

20   Tropical Medicine and Hygiene*; 39(4), 1988, p380-387. It is known in this model

that *D. immitis* antigen appears in the blood approximately seven-months post-

infection. For this example, animals were infected with *D. immitis* and urine samples

collected monthly by catheterization. It should be noted that in some cases, the

process of catheterization can result elevated albumin levels; as a resul, animals were

25   only considered positive for micoalbuminuria when they were found to be

microalbuminuric in two consecutive samples. The amount of albumin in each sample was determined using an ELISA assay. The results are shown below in Table 11. Boxes labeled N/A indicate where no sample was available.

Table 11

Months Post Infection	Animal Identifier											
	HOP (A)	IGH (A)	POR (A)	SSH (A)	XTJ (A)	YOH (A)	AXH (B)	CAH (B)	FVH (B)	GUH (B)	HOH (B)	VIP (B)
	174.9	0.2	2.3	1.9	0.3	2.9	8.0	72.6	0.3	3.7	4.2	2.1
1	2.1	3.8	2.4	0.3	N/A	8.7	4.0	4.0		3.7	0.2	0.4
2	33.8	2.0	9.5	2.4	29.9	N/A	3.3	0.2	3.0	3.3	0.9	2.6
4	1.3	16.4	0.3	3.0	0.3	0.3	3.7	22.7	2.5	2.3	1.8	5.4
5	2.2	4.2	N/A	2.0	18.1	4.2	3.4	3.0	0.3	1.9	0.2	4.1
6	4.9	9.9	N/A	2.5	N/A	0.3	0.2	2.6	2.7	1.5	0.4	0.4
7	1.4	48.1	0.3	0.3	20.7	45.6	3.9	0.3	1.9	20.7	3.3	8.1
8	8.4	N/A	18.0	N/A	60.6	16.2	3.1	4.8	6.5	2.8	8.5	15.4
9	26.2	2.9	4.4	0.4	46.8	16.1	6.0	0.3	24.6	3.6	0.3	N/A
10	N/A	11.0	3.4	10.8	26.2	15.7	13.1	21.3	54.0	60.3	0.2	46.0
11	52.1	125.7	43.5	36.9	180.6	67.8	3.9	27.3	11.5	6.5	59.5	736.
12	58.5	16.2	22.2	52.9·	51.3	54.9	6.8	76.2	23.4	5.9	97.4	167.2
13	113.5	56.4	25.1	8.1	132.4	112.7	14.7	30.1	327.2	13.3	65.5	132.6
14	134.3	60.2	132.1	16.8	123.0	82.9	66.6	65.8	500.0	5.0	285.7	69.06.1
15	206.0	4.0	122.0	23.7	39.1	18.6	3.8	16.4	500.0	8.0	107.8	34.8
16	37.3	7.6	500.0	5.4	52.5	10.1	5.5	17.8	500.0	4.9	43.1	N/A
17	N/A	45.2	N/A	8.6	181.6	89.1	30.9	19.8	500.0	16.4	53.0	N/A
18	18.8	112.1	211.1	5.4	70.4	26.8	10.5	14.9	N/A	5.4	21.3	N/A
19	16.7	67.0	176.7	12.1	208.4	N/A	N/A	36.3	N/A	4.9	57.1	N/A
20	N/A	N/A	N/A	N/A	500.0	N/A	N/A	N/A	N/A	1.9	N/A	N/A
21	N/A	N/A	N/A	N/A	N/A	37.9	9.2	41.7	N/A	11.0	17.1	N/A
22	1.6	4.7	75.8	9.1	500.0	27.2	22.2	500.0	N/A	3.2	46.2	N/A
23	83.2	37.6	30.3	17.5	500.0	60.3	54.3	500.0	N/A	5.2	37.7	N/A

The data demonstrate that following infection with *D. immitis*, there is a progressive increase in the level of albumin in the urine. Additionally, most animals became microalbuminuric within 1-2 months following the time of appearance of *D. immitis* antigen in the blood. Microalbuminuria could be detected in all animals by the end of the study.

Example 16

Albumin levels in canines suffering from hereditary nephritis

This example compares the level of microalbuminuria (MA) with a commonly used marker for renal disease, the urinary protein/ creatinine (UP/C) ratio, over time in animals suffering from hereditary nephritis (HD). In this model, the animals carry a genetic defect which results in the rapid development of renal disease during the course of the animals life as described in Lee, GE, *American Journal of Veterinary Research*, 1999: 60, p373-383. In this example, urine was periodically collected from

5   a colony of normal dogs and a colony of dogs suffering HD. The amount of albumin

in each sample was determined using an ELISA assay. In addition, the urinary

protein/ creatinine UP/C) ratio was determined using veterinary reference lab. By

this measurement, renal disease is considered to be present when the UP/C ratio is

greater than 1.0. The results of this study are shown below in Table 12.

10

Table 12

Age (weeks)	Fonzi (control) UP/C Ratio	Fonzi (control) MA (μg/ml)	Jake (control) UP/C Ratio	Jake (control) MA	Ned (control) UP/C Ratio	Ned (control) MA (μg/ml)	Oscar (control) UP/C Ratio	Oscar (control) MA (μg/ml)	Pete (control) UP/C Ratio	Pete (control) MA (μg/m
8	0.1	2	1.6	0	0.2	5	0.6	2	0.9	3
11	0.2	2	0.7	5	0.2	5	0.2	8	0.3	4
13	0.3	1	0.3	0	0.2	5	0.9	3	0.4	2
15	1.0	3	0.6	6	0.2	5	0.3	4	0.2	2
17	0.2	1	0.2	3	0.2	5	0.1	3	0.2	6
19	0.4	15	0.5	4	0.2	5			0.1	3
21	0.1	4	1.0	7	0.2	5	0.1	2	0.1	1
23	0.3	0	0.2	3	0.2	5	0.2	1	0.1	1
25	0.6	1	0.1	6	0.2	5	0.1	1	0.1	20
27	0.1	-1	0.2	6	0.2	5	0.1	0	0.1	6
30	0.1	2	0.1	4	0.2	5	0.1	2	0.0	1
34	0.2	220	0.1	4	0.2	5	0.1	1	0.1	2
38	0.1	1	0.1	2	0.2	5	0.1	1	0.1	4

Age (weeks)	Ethan (HN) UP/C Ratio	Ethan (HN) MA (μg/ml)	Frasier (HN) UP/C Ratio	Frasier (HN) MA (μg/ml)	Greg (HN) UP/C Ratio	Greg (HN) MA (μg/ml)	Ike (HN) UP/C Ratio	Ike (HN) MA (μg/ml)	Lester (HN) UP/C Ratio	Lester (HN) MA (μg/m
8	0.2	4	0.1	6	0.1	2	1.0	6	0.8	10
11	0.3	9	0.2	4	0.1	4	0.2	10	0.4	8
13	0.6	4	0.2	1	0.3	2	0.2	1	0.5	12
15	0.5	8	0.3	12	0.1	12	0.7	7	0.3	3
17	0.1	17	0.6	358	0.2	487	1.0	557	0.4	7
19	1.0	82	2.3	314	0.4	2	4.4	918	0.6	115
21	3.0	136	1.1	30	0.2	2	6.6	1574	1.0	561
23	6.2	4954	5.1	2145	0.3	71	12.5	5560	3.0	615
25	10.1	744	9.0	3000	1.6	603	16.6	2920	3.7	17
27	6.6	1179	7.3	2020	3.5	1499	15.3	3904	7.0	1477
30	15.7	2734	12.3	2696	5.7	1733	16.5	2276	9.3	1679
34	11.6	1901	12.9	2	8.2	309	4.4	3608	8.7	1992
38	6.4	3310	13.9	3597	8.8	4845	8.5	4465	8.1	1919

Age (weeks)	Nate (HN) UP/C Ratio	Nate (HN) MA (μg/ml)	Newt (HN) UP/C Ratio	Newt (HN) MA (μg/ml)	Quark (HN) UP/C Ratio	Quark (HN) MA (μg/ml)	Quirt (HN) UP/C Ratio	Quirt (HN) MA (μg/ml)	Eddie (HN) UP/C Ratio	Eddie (HN) MA (μg/m
8	0.4	6	0.2	5	0.1	2	0.4	1	1.6	16
11	0.4	0	0.4	0	0.1	3	0.4	4	0.2	10
13	0.4	5	0.2	4	0.7	11	0.2	3	0.4	6
15	0.4	2	0.1	19	0.3	5	0.1	1	0.3	6
17	0.3	4	1.1	116	0.4	74	0.1	12	0.1	5
19	0.6	7	1.5	265	0.6	232	0.2	25	0.4	10
21	0.2	52	2.4	1321	2.8	620	0.6	267	1.2	1063
23	2.1	340	8.7	2665	9.2	1223	3.4	543	2.8	1307
25	2.2	622	9.6	4711	8.8	1938	4.6	1208	8.7	1947
27	3.2	483	10.1	1309	7.8	2007	9.1	3054	6.9	1052
30	5.8	1529	9.0	2989	14.1	3419	9.3	2747	13.9	3188
34	7.3	1483	8.8	1806	13.1	3055	9.9	6379	10.5	4927
38	8.9	2955	8.1	6487	12.2	3118	9.5	3044	12.7	6717

Age (weeks)	Felix (HN) UP/C Ratio	Felix (HN) MA (μg/ml)	Fred (HN) UP/C Ratio	Fred (HN) MA (μg/ml)	Gus (HN) UP/C Ratio	Gus (HN) MA (μg/ml)	Neal (HN) UP/C Ratio	Neal (HN) MA (μg/ml)	Norm (HN) UP/C Ratio	Norm (HN) MA (μg/m
8	0.7	1	0.3	3	0.1	5	0.8	0	0.3	1
11	0.1	8	0.1	12	0.1	4	0.2	3	0.1	1
13	0.1	1	0.5	1	0.1	22	0.4	3	0.1	0
15	0.3	5	0.6	1	0.2	55	0.1	2	0.5	1

17	0.8	122	0.5	6	1.7	24	0.4	2	0.7	4
19	0.3	87	0.3	13	2.2	77	0.6	428	0.5	7
21	0.8	903	0.8	9	3.9	16	0.6	210	1.3	354
23	2.6	1679	0.6	81	9.3	1565	6.6	1335	5.7	1535
25	6.9	16170	1.9	152	6.4	3950	8.4	4091	9.5	3290
27	10.2	2452	3.5	11	5.2	1263	10.1	1158	5.5	798
30	12.0	2612	8.1	1887	8.3	2648	9.2	2523	6.8	2796
34	9.3	4146	7.1	3403	8.5	4583	10.1	1767	11.5	2603
38	10.4	6218	10.8	7141	7.9	3758	10.4	2906	7.0	3403

	Paul (HN)		Quinn (HN)		Scooter (HN)					
Age (weeks)	UP/C Ratio	MA (μg/ml)	UP/C Ratio	MA (μg/ml)	UP/C Ratio	MA (μg/ml)	UP/C Ratio	MA (μg/ml)	UP/C Ratio	MA (μg/m
8	0.6	4	0.1	5	1.4					
11	0.1	8	0.1	1	0.2	3				
13	0.4	1	0.2	5	0.3	7				
15	0.2	0	0.2	1	0.1	21				
17	0.1	6	0.1	3	0.3	66				
19	0.7	6	0.6	5	2.7	323				
21	0.1	58	0.1	16	4.3	20				
23	1.3	206	0.6	29	8.8	2678				
25	2.0	598	1.5	224	11.3	2957				
27	4.2	674	2.1	431	10.1	3864				
30	4.0	2650	5.4	1468	11.2	2118				
34	5.5	0	10.0	1395	12.5	5098				
38	6.4	4324	8.6	1624	8.4	3238				

The data demonstrate that there is a progressive increase in microalbuminuria in animals suffering from hereditary nephritis. In addition, in virtually all animals, micoralbuminuria was detected prior to the UP/C ration being greater than 1.0.

Although the invention has been described with reference to the presently preferred embodiments, it should be understood to those skilled in the art that various modifications can be made without departing from the spirit of the invention. Accordingly, the invention is limited only by the following claims.

5   What is claimed is:

        1.   A method to detect early renal disease comprising:

            (a)   obtaining a sample from an animal; and

            (b)   determining the amount of albumin in said sample,

            wherein an amount of albumin in a range of from about 10 μg/ml to

10   about 300 μg/ml in the sample, when the specific gravity of the sample is normalized

to 1.010 g/ml, is indicative of early renal disease.

        2.   A method to identify an animal at risk for developing late-stage renal

disease comprising:

            (a)   obtaining a sample from the animal; and

15   (b)   determining the amount of albumin in said sample,

            wherein an amount of albumin in a range of about 10 μg/ml to about

300 μg/ml in the sample, when the specific gravity of the sample is normalized to

1.010, indicates the animal is at risk for late-stage renal disease.

        3.   A kit comprising a means for detecting early renal disease according to

20   the method of claim 1.

        4.   A kit comprising a means for detecting early renal disease according to

the method of claim 2.

        5.   A kit comprising:

            (a)   an albumin-binding compound which selectively binds to

25   animal albumin; and

            (b)   a means for detecting the amount of albumin in a sample

collected from an animal, wherein said means detects albumin in the sample when the

amount of albumin is in the range of from about 10 μg/ml to about 50 μg/ml.

        6.   The kit of claim 3 comprising:

30   (a)   an albumin-binding compound which selectively binds to

animal albumin; and

            (b)   a means for assessing the amount of albumin in the sample

when the amount of albumin is in the range of 10 μg/ml to 300 μg/ml when the

specific gravity of the sample is normalized to 1.010 g/ml.

35   7.   The kit of claim 4 comprising:

            (a)   an albumin-binding compound which selectively binds to

animal albumin; and

(b)   a means for assessing the amount of albumin in the sample when the amount of albumin is in the range of 10 μg/ml to 300 μg/ml when the specific gravity of the sample is normalized to 1.010 g/ml.

8.   An isolated monoclonal antibody which selectively binds albumin of a specie of animal selected from the group consisting of canids, felids and equids.

9.   A cultured cell which produces an antibody that selectively binds albumin of an animal selected from the group consisting of canids, felids and equids.

10.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the amount of albumin in the sample is determined by:

(a)   contacting the sample with an albumin-binding compound to form an albumin-compound complex;

(b)   detecting the albumin-compound complex; and

(c)   assessing the amount of albumin present in the sample from the amount of albumin-compound complex detected.

11.   The invention of claim 10, wherein the albumin-binding compound is an anti-albumin antibody.

12.   The invention of claim 10, wherein the albumin-binding compound is an anti-albumin antibody which recognizes albumin from an animal selected from the group consisting of canids, felids and equids.

13.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the amount of albumin in the sample is determined using an assay selected from the group consisting of an enzyme-linked immunoassay, a radioimmunoassay, a fluorescence immunoassay, a chemiluminescent assay, a lateral-flow assay, a dipstick assay, an agglutination assay, a particulate-based assay, an immunoprecipitation assay, an immunodot assay, an immunoblot assay, an immunodiffusion assay, a phosphorescence assay, a flow-through assay, a chromatography assay, a PAGe-based assay, an electronic-sensory assay, a surface plasmon resonance assay and a fluorescence correlation spectroscopy assay.

14.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the amount of albumin in the sample is determined using an enzyme-linked immunosorbent assay (ELISA).

15.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the amount of albumin in the sample is determined using a single-step assay.

5   16.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the amount of albumin in the sample is determined using a dipstick-based assay.

       17.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the amount of albumin is determined using an assay that detects albumin in the sample when the amount of albumin is in the range from about 10 µg/ml to about 25 µg/ml.

10   18.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the amount of albumin is determined using an assay that detects albumin in the sample when the amount of albumin is about 50 µg/ml.

       19.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the amount of albumin is determined using an assay that detects albumin in the sample when the

15 amount of albumin is about 25 µg/ml.

       20.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the amount of albumin is determined using an assay that detects albumin in the sample when the amount of albumin is about 10 µg/ml.

       21.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the animal is

20 selected from the group consisting of canids, felids and equids.

       22.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the animal is a canid.

       23.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the animal is a felid.

25   24.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the animal is an equid.

       25.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the sample is a bodily fluid useful for measuring leakage of albumin from the glomerulus.

       26.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the sample is urine.

30   27.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the sample is pre-treated prior to determining the amount of albumin.

       28.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the sample is pre-treated by adjusting the specific gravity.

       29.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the sample is pre-

35 treated by adjusting the specific gravity to 1.010 g/ml.

       30.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the amount of albumin is determined using a single-step assay.

5   31.   The invention of claim 1, 2, 3, 4, 5, 6 or 7, wherein the amount of albumin is determined using a dipstick assay or an ELISA assay.

           32.   The antibody of claim 8 or 9, wherein the antibody inhibits the selective binding of an antibody selected from the group consisting of H352, H386, H387, H388, H389, H390, H391, H393, H394, H395, H396, H397, H398, H399,

10   H400, H401 and H402 to albumin.

           33.   The antibody of claim 8 or 9, wherein the antibody binds the same epitope recognized by an antibody selected from the group consisting of H352, H386, H387, H388, H389, H390, H391, H393, H394, H395, H396, H397, H398, H399, H400, H401 and H402.

15   34.   The isolated antibody of claim 8 or 9, wherein the antibody is selected from the group consisting of H352, H386, H387, H388, H389, H390, H391, H393, H394, H395, H396, H397, H398, H399, H400, H401 and H402.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/11105

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(7) : GOIN 33/53, 31/00, 33/00
US CL : 435/7.1, 7.95; 436/2, 86, 88

According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/7.1,7.95, 436/286,88

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
STN

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,246,835 A (SUZUKI ET AL) 21 September 1993 (21.09.1993), (Summary of Invention); column 1, lines 36-47; column 3, lines 65-68 and col 4, lines 3-8;column 10, lines 10-62; column 2, lines 56-68 and column 3, lines 1-8; column 10, lines 51-54; See abstract; column 4, lines 45-68.	1, 2-7, 8, 9, 10-12, 21-26, 27
Y	US 6,152,392 A (LIAO ET AL) 28 November 2000 (28.11.2000), column 19, lines 65-67 and column 20, lines 1-36; column 21, lines 14-31.	13-20, 30-31
Y	US 5,403,744 A (ZIMMERLE) 04 April 1995 (04.04.1995), see abstract.	28-29

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:
"A"	document defining the general state of the art which is not considered to be of particular relevance
"E"	earlier application or patent published on or after the international filing date
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"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 July 2002 (28.07.2002)	12 SEP 2002

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